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SUSCEPTIBILITY

Toward Harmonization of Voriconazole CLSI and EUCAST Breakpoints for *Candida albicans* Using a Validated *In Vitro* Pharmacokinetic/Pharmacodynamic Model

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ABSTRACT CLSI and EUCAST susceptibility breakpoints for voriconazole and *Candida albicans* differ by one dilution (≤0.125 and ≤0.06 mg/liter, respectively) whereas the epidemiological cutoff values for EUCAST (ECOFF) and CLSI (ECV) are the same (0.03 mg/liter). We therefore determined the pharmacokinetic/pharmacodynamic (PK/PD) breakpoints of voriconazole against *C. albicans* for both methodologies with an *in vitro* PK/PD model, which was validated using existing animal PK/PD data. Four clinical wild-type and non-wild-type *C. albicans* isolates (voriconazole MICs, 0.008 to 0.125 mg/liter) were tested in an *in vitro* PK/PD model. For validation purposes, mouse PK were simulated and *in vitro* PD were compared with *in vivo* outcomes. Human PK were simulated, and the exposure-effect relationship area under the concentration-time curve for the free, unbound fraction of a drug from 0 to 24 h ($fAUC_{0-24}$)/MIC was described for EUCAST and CLSI 24/48-h methods. PK/PD breakpoints were determined using the $fAUC_{0-24}$/MIC associated with half-maximal activity (EI_{50}) and Monte Carlo simulation analysis. The *in vitro* 24-h PD EI_{50} values of voriconazole against *C. albicans* were 2.5 to 5 (1.5 to 17) *f*AUC/MIC. However, the 72-h PD were higher at 133 (51 to 347) *f*AUC/MIC for EUCAST and 94 (35 to 252) *f*AUC/MIC for CLSI. The mean (95% confidence interval) probability of target attainment (PTA) was 100% (95 to 100%), 97% (72 to 100%), 83% (35 to 99%), and 49% (8 to 91%) for EUCAST and 100% (97 to 100%), 99% (85 to 100%), 91% (52 to 100%), and 68% (17 to 96%) for CLSI for MICs of 0.03, 0.06, 0.125, and 0.25 mg/liter, respectively. Significantly, >95% PTA values were found for EUCAST/CLSI MICs of ≤0.03 mg/liter. For MICs of 0.06 to 0.125 mg/liter, trough levels 1 to 4 mg/liter would be required to attain the PK/PD target. A PK/PD breakpoint of *C. albicans* voriconazole at the ECOFF/ECV of 0.03 mg/liter was determined for both the EUCAST and CLSI methods, indicating the need for breakpoint harmonization for the reference methodologies.

KEYWORDS voriconazole, *Candida albicans*, susceptibility breakpoints, CLSI, EUCAST, PK/PD, antifungal susceptibility testing, breakpoints

Bloodstream infections caused by *Candida* species are an important public health problem and are associated with significant morbidity and mortality, increased lengths of hospital stay, and significant economic burden (1, 2). The emergence of azole resistance among *Candida* spp. is of particular concern, especially in cases with prior

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TABLE 1 MICs for EUCAST and CLSI for *Candida* isolates used in the present study

C. albicans isolate no.	Reference code	Median (range) MIC		
		EUCAST	CLSI24	CLSI48
1	K1	0.008 (0.004–0.008)	0.008 (0.008–0.016)	0.008 (0.008–0.016)
2	2-76	0.008 (0.008–0.016)	0.016 (0.008–0.03)	0.016 (0.016–0.06)
3	1490	0.016 (0.008–0.016)	0.016 (0.016–0.03)	0.03 (0.016–0.03)
4	9817	0.125 (0.06–0.125)	0.125 (0.06–0.25)	0.25 (0.06–0.5)

exposure to fluconazole (3). Thus, it becomes evident that antimicrobial susceptibility testing for clinical management of these infections as well as antimicrobial resistance surveillance becomes of great importance.

Both EUCAST and CLSI have standardized their methodologies for antifungal susceptibility testing with similar testing conditions (4). A great effort was made in order to harmonize the clinical breakpoints for the two methods, particularly for antifungal drugs for which similar MIC distributions are generated (5). The use of 24-h MICs and species-specific breakpoints by the CLSI had led to harmonization of fluconazole clinical breakpoints between the two reference methodologies (5). The similar voriconazole MIC distribution (determined with 24 h of incubation) and published epidemiological cutoff values by CLSI and EUCAST of ≤0.03 mg/liter for *Candida albicans* (6, 7) confirms the comparability of the two methods for voriconazole susceptibility testing. EUCAST has recently revised the voriconazole epidemiological cutoff (ECOFF) based on newer larger data sets from 0.125 mg/liter to 0.03 mg/liter (6). In order to account for the lower ECOFF compared to those of previous susceptibility breakpoints, EUCAST in 2017 has decreased voriconazole breakpoints by one 2-fold dilution from susceptible (S) ≤ 0.125/resistant (R) > 0.5 mg/liter to S ≤ 0.06/R > 0.25 mg/liter (6), whereas CLSI breakpoints are S ≤ 0.125/R > 0.5 mg/liter (8). This may lead to artificially differential resistance rates despite the two methods generating similar MICs (9). More importantly, both breakpoints are higher than corresponding EUCAST ECOFFs and CLSI epidemiological cutoff values (ECVs), indicating that non-wild-type isolates can be treated with voriconazole.

We therefore determined pharmacokinetic/pharmacodynamic (PK/PD) breakpoints for voriconazole and *C. albicans* for EUCAST and CLSI using an *in vitro* PK/PD model where human voriconazole pharmacokinetics were simulated (10) after the model was validated with *C. albicans* isolates with increasing voriconazole MICs previously used in animal PK/PD studies (11).

RESULTS

MICs. The MICs of all strains with EUCAST after 24 h and CLSI after 24 h (CLSI24) and 48 h (CLSI48) are shown in Table 1. Most MICs (except 1 *C. albicans* strain) among methods were within one 2-fold dilution, with higher absolute agreement found between EUCAST and CLSI24 MICs (64%) than CLSI48 MICs (46%) since all discrepant CLSI48 MICs were one 2-fold dilution higher than EUCAST MICs.

Simulation of the mouse model. (i) Pharmacokinetics. Mouse pharmacokinetics of 10, 20 and 40 mg of drug/kg of body weight/day voriconazole dosages were well simulated in the *in vitro* model with attained maximum concentration (C_{max}) values (mean ± standard deviation [SD]) of 0.40 ± 0.11, 1.56 ± 0.31, and 6.74 ± 1.18 mg/liter and area under the concentration-time curve values from 0 to 24 h (AUC_{0-24}) of 1.1 ± 0.33, 5.0 ± 0.95, and 25.6 ± 4.4 mg · h/liter, respectively, and a mean ± SD half-life ($t_{1/2}$) of 2.78 ± 0.94 h.

(ii) Pharmacodynamics. In drug free controls, fungal load increased from 3.79 ± 0.10 $\log_{10}$ CFU/ml at $t = 0$ h to 7.25 ± 0.59 $\log_{10}$ CFU/ml at $t = 24$ h (as observed also in animals) and 7.76 ± 0.6 $\log_{10}$ CFU/ml at $t = 48$ h. The maximum reduction of fungal load in drug-treated tubes compared to drug-free controls at 24 and 48 h was ~3 $\log_{10}$ CFU/ml corresponding to a 1 $\log_{10}$ CFU/ml increase from the initial inoculum as previously observed also in animals (11) (Fig. 1). No killing was observed compared

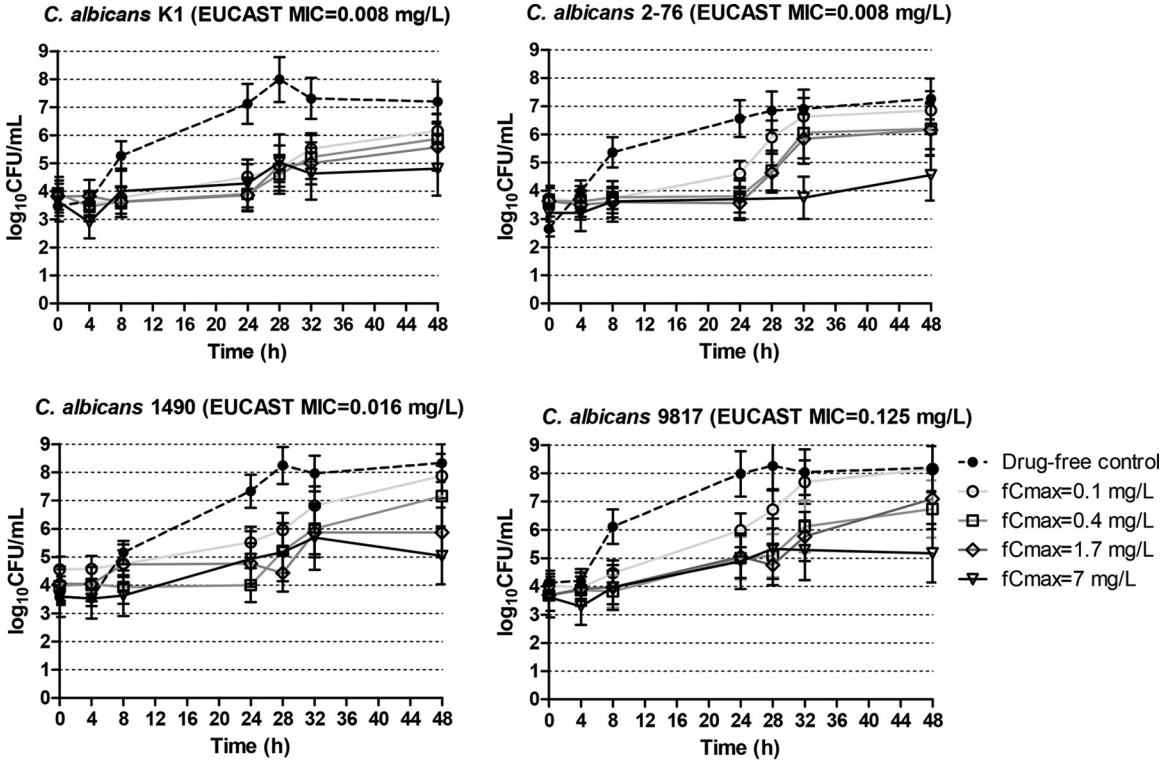

FIG 1 Time-kill curves in the *in vitro* PK/PD model simulating animal every 24 h (q24h) oral dosing regimens of voriconazole against *C. albicans* isolates targeting different fC_{max} values with $t_{1/2}$ values of 2 to 4 h. Error bars represent SD.

to the fungal burden at the start of therapy at any voriconazole simulated dosages as in animals (11). The *in vitro* exposure-effect relationship for the *C. albicans* isolates followed a sigmoid curve (R^2 = 0.88 to 0.91) for both the 24-h and 48-h pharmacodynamics with EUCAST and CLSI MICs (Fig. 2).

(iii) Comparison between *in vitro* and *in vivo* pharmacodynamics. The mean (95% confidence interval [CI]) CLSI half-maximal activity (EI_{50}) in the *in vitro* model was 2.8 (1.5 to 5.5) area under the concentration-time curve for the free, unbound fraction of voriconazole from 0 to 24 h ($fAUC_{0-24}$)/MIC, whereas the *in vivo* EI_{50}s found previously in animals for the same *C. albicans* isolates were 13.3 to 25.3 $fAUC_{0-24}$/MIC (11). The mean (95% CI) EI_{50} using EUCAST MICs was 2.5 (1.5 to 4.5) $fAUC_{0-24}$/MIC. Due to growth of *C. albicans* after 24 h, the 48 h EI_{50} was higher than the 24 h EI_{50}, reaching a mean (95% CI) of 40 (7.5 to 211) $fAUC_{0-24}$/MIC for CLSI and 40 (9 to 186) $fAUC_{0-24}$/MIC for EUCAST (Fig. 2).

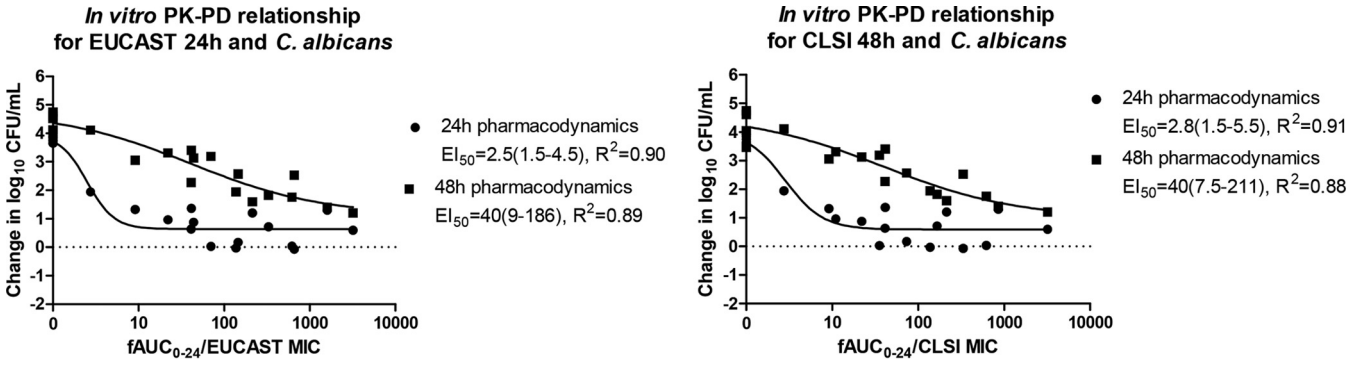

FIG 2 *In vitro* PK/PD relationship of voriconazole against *C. albicans* as a function of change in log_{10}CFU/ml from initial fungal load and $fAUC_{0-24}$/MIC simulating animal PKs.

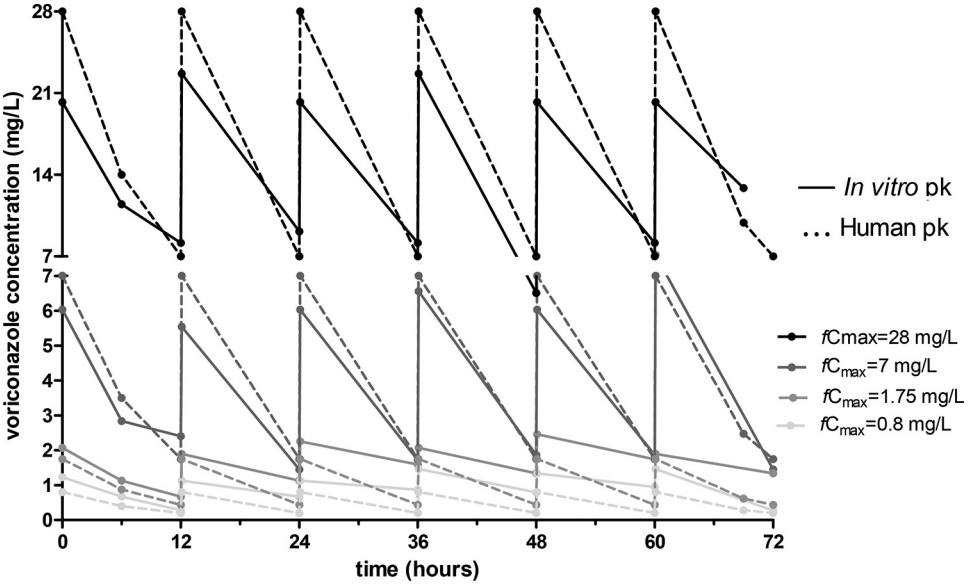

FIG 3 Representative time-concentration profiles of simulated every 12 h (q12h) i.v. dosing regimens of voriconazole in the *in vitro* PK/PD model with target C_{max} values of 0.8, 1.75, 7, and 28 mg/liter and obtained C_{min} values of 0.28, 0.67, 1.04, and 1.75 mg/liter, respectively, and a $t_{1/2}$ of 6 h. Data represent drug levels in the internal compartment of the *in vitro* model (solid lines) and the respective target values (broken lines).

Pharmacokinetics. Steady-state human plasma pharmacokinetics of twice daily voriconazole dosages were well-simulated in the *in vitro* PK/PD model. The mean ± SD $t_{1/2}$ was 7.9 ± 1.6 h with free-drug concentration (fC_{max}) values of 20.05 ± 0.06, 5.29 ± 0.04, 2.06 ± 0.15, and 1.35 ± 0.12 mg/liter and fAUC$_{0\text{-}24}$ values of 342.89 ± 9.48, 88.31 ± 3.56, 42.41 ± 2.63, and 27.17 ± 2.37 mg · h/liter, respectively (Fig. 3).

Pharmacodynamics. For *C. albicans*, the fungal load increased from 4.12 ± 0.24 $\log_{10}$ CFU/ml at $t = 0$ h to 8.37 ± 0.26 $\log_{10}$ CFU/ml at $t = 72$ h in drug-free controls (Fig. 4). Over the range of voriconazole doses studied, no killing of organisms was observed for any of the *C. albicans* isolates compared to initial inoculum. The maximum effect corresponded to 1 $\log_{10}$ CFU/ml increase from initial inoculum. The *in vitro* exposure-effect relationship for the *C. albicans* isolates followed a sigmoid curve (R^2 = 0.86 to 0.89). Curve fits of EUCAST- and CLSI24/48-derived methods were comparable with mean (95% CI) EI$_{50}$s of 133 (51 to 347) and 94 (35 to 252)/96 (44 to 208) fAUC$_{0-24}$/MIC, respectively (Fig. 5). Notably, the EI$_{50}$ increased over time for all three methods (EUCAST, CLSI24, CLSI48) from 3.6 to 5 fAUC$_{0\text{-}24}$/MIC after 24 h to 37 to 53 fAUC$_{0\text{-}24}$/MIC after 48 h and 94 to 133 fAUC$_{0\text{-}24}$/MIC after 72 h for CLSI and EUCAST, respectively (Fig. 6), similar to the increase of EI$_{50}$ over time found when mouse serum pharmacokinetics were simulated (see above).

Monte Carlo analysis. The simulated patients had a mean ± SD fAUC$_{0-24}$ of 41.94 ± 35.41 mg · h/liter, which is very close to previously published voriconazole exposures (12). The mean (95% CI) percent probabilities of target attainment (PTAs) for EUCAST PK/PD target 133 (51 to 347) fAUC$_{0-24}$/MIC were 100 (95 to 100), 97 (72 to 100), 83 (35 to 99), 49 (8 to 91), 16 (1 to 6), and 2 (0 to 27) for EUCAST MICs of 0.03, 0.06, 0.125, 0.25, 0.5, and 1 mg/liter, respectively. Similar analysis for CLSI24 PK/PD target 94 (35 to 252) fAUC$_{0-24}$/MIC, the mean (95% CI) percent probability of target attainment (PTA) was 100 (97 to 100), 99 (85 to 100), 91 (52 to 100), 68 (17 to 96), 31 (3 to 81), and 6 (0 to 46) for CLSI MICs of 0.03, 0.06, 0.125, 0.25, 0.5, and 1 mg/liter, respectively (Fig. 6). The PTAs were significantly higher than 95% and 50% for EUCAST/CLSI MICs of ≤0.03 and 0.06 to 0.125 mg/liter, respectively.

Trough levels and MICs. The voriconazole trough levels in human serum required to attain the corresponding PK/PD targets for *C. albicans* isolates with increasing EUCAST and CLSI24 MICs are shown in Fig. 7. The corresponding PK/PD targets could

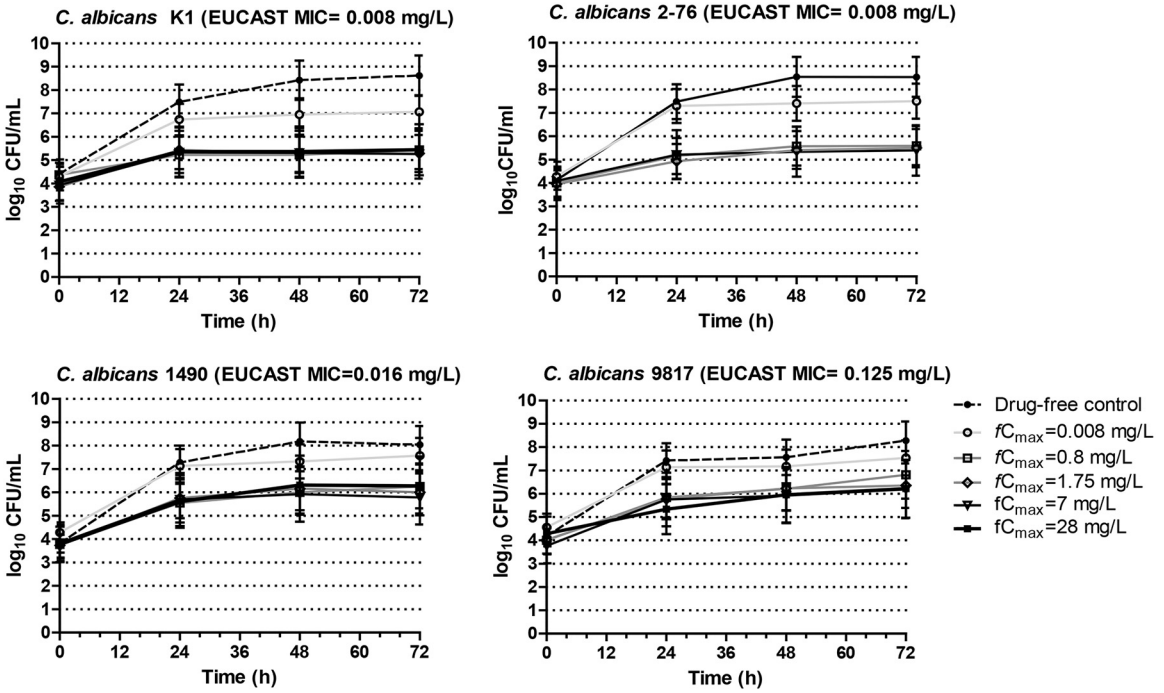

FIG 4 Time-kill curves in the *in vitro* PK/PD model simulating human q12h i.v. dosing regimens of voriconazole against *C. albicans* isolates with fC_{max} values of 0.008, 0.8, 1.75, 7, and 28 mg/liter and a $t_{1/2}$ of 6 h. Error bars represent SD.

be attained for *C. albicans* isolates with EUCAST/CLSI24 MICs of ≤0.03 mg/liter, whereas for isolates with MICs of 0.06 to 0.125 mg/liter, upper 95% CI trough levels of 1 to 4 mg/liter may be required. In contrast, isolates with higher MICs will require trough levels of ≥4 mg/liter, which is difficult to achieve and usually associated with increased risk of toxicity (13).

DISCUSSION

An *in vitro* PK/PD model was used to determine PK/PD breakpoints for voriconazole and *C. albicans* for EUCAST and CLSI reference methods. The model was first validated using the same *C. albicans* isolates previously used in an animal neutropenic model of disseminated candidiasis simulating animal voriconazole pharmacokinetics. Simulating human pharmacokinetics, similar 24-h PK/PD indices were found, but higher 48-h and 72-h PK/PD indices were determined for EUCAST and CLSI (133 [51 to 347] and 94 [35 to 252] $fAUC_{0-24}$/MIC, respectively). Based on the latter PK/PD indices, the following PK/PD breakpoints were determined for EUCAST and CLSI: ≤0.03, 0.06 to 0.125, and ≥0.25 mg/liter, suggesting that lowering the current CLSI and EUCAST S breakpoints by

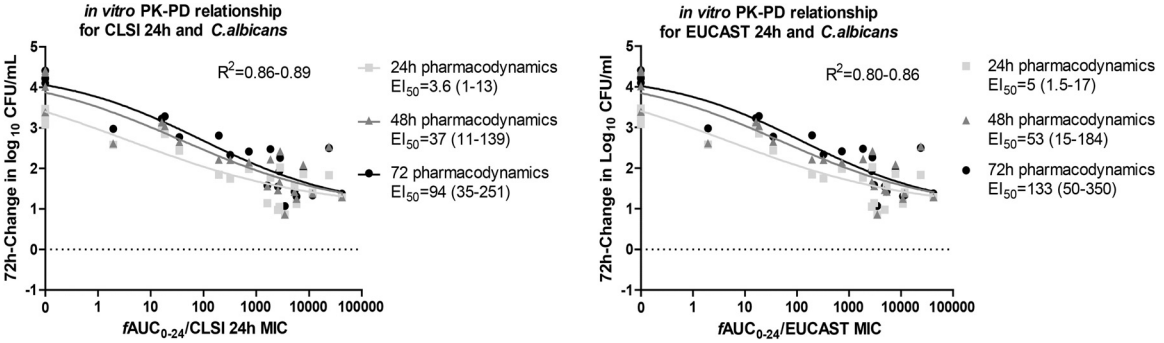

FIG 5 *In vitro* PK/PD relationship of voriconazole against *C. albicans* as a function of 72-h change in $\log_{10}$ CFU/ml from initial fungal load (horizontal dotted line) and $fAUC_{0-24}$/MIC simulating human PKs.

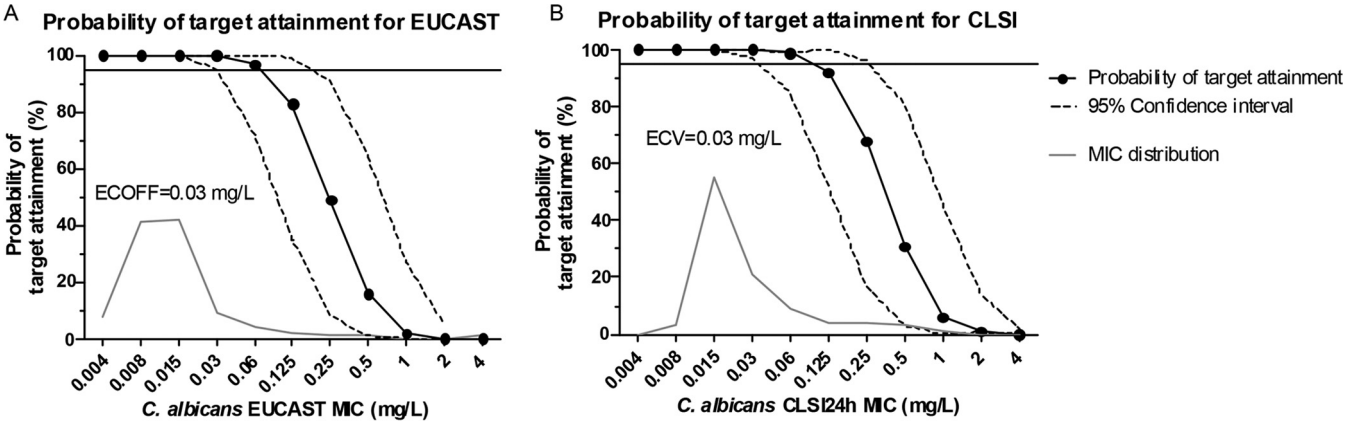

FIG 6 Target attainment rates for 1,000 patients receiving standard voriconazole dosage of 4 mg/kg i.v. twice daily for which the AUCs were simulated with Monte Carlo for different EUCAST and CLSI 24-h MICs. Horizontal line represents the 95% PTA.

two and one 2-fold dilution would be appropriate in order to (i) attain high PTAs for wild-type isolates (MIC $\leq$ 0.03 mg/liter), (ii) classify non-wild-type isolates with low MICs (0.06 to 0.125 mg/liter) as intermediate, i.e., the PK/PD targets can be achieved only if sufficient exposure is attained, (iii) avoid using voriconazole for non-wild-type isolates with the higher MICs ($\geq$0.25 mg/liter) since the PK/PD target for those isolates can be attained with confidence with trough levels of $\geq$4 mg/liter associated with high toxicity, and (iv) harmonize voriconazole breakpoints between the two methodologies as previously done for fluconazole.

In line with previous *in vitro* PK/PD studies, voriconazole demonstrated concentration-independent pharmacodynamics, arresting growth without any killing of *C. albicans* even at very high concentrations (14). Lack of killing was also observed in a neutropenic mouse model of experimental disseminated candidiasis with a maximum organisms' reduction from untreated animals after 24 h of up to 3 $\log_{10}$ CFU/kidney (11), which corresponds to a 1 $\log_{10}$ CFU/kidney increase from initial inoculum as in the present study. Animal PK/PD studies indicated a strong relationship for AUC/MIC with an R^2 of 82%, similar to the present study. The 24-h PK/PD target associated with 50% of maximal activity (EI_{50}), corresponding to an $\sim$2 $\log_{10}$ increase of fungal load from initial inoculum (or decrease from untreated animals at 24 h), was 13.3 to 25.3 *f*AUC/MIC for CLSI, taking into account the 78% protein binding in mouse serum (11). The *in vitro* mean (95% CI) 24-h EI_{50} found in the present study was slightly lower at 2.5 to 5 (1.5 to 17) *f*AUC/MIC, possibly related to an absence of host-related factors. Of note, drug toxicity (observed at >80 mg/kg) in mice could affect *in vivo* pharmacodynamics (11).

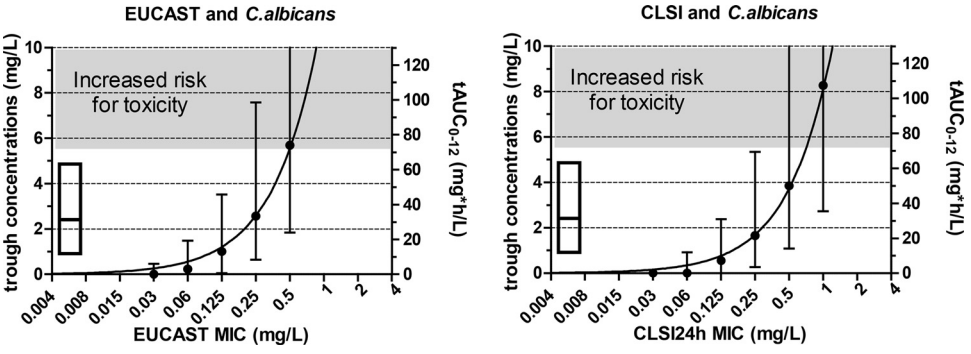

FIG 7 Correlation between voriconazole trough concentrations in human serum and EUCAST/CLSI24 MICs of *C. albicans* in order to attain the corresponding PK/PD targets of 133 (51 to 347) for EUCAST and 94 (35 to 252) for CLSI24, respectively. Median and interquartile range trough levels are shown for 250 patients treated with different doses of voriconazole (18).

However, the 24-h period was not enough time to describe the pharmacodynamics of voriconazole because maximum growth in drug-free control was not achieved and regrowth occurred in drug-treated tubes after 24 h. Consequently, the PK/PD indices associated with 50% efficacy at 48 and 72 h increased 4 times compared to those at 24 h. *Candida* regrowth after 24 h of drug exposure has also been previously observed in an *in vitro* PK/PD model with fluconazole (even at high concentrations) and caspofungin (at concentrations close to MIC) (15).

The animal 24-h PK/PD target of 25 *f*AUC/MIC results in high PTAs in Monte Carlo analysis for isolates with MICs up to 1 mg/liter, which is 8-fold higher than the current susceptibility CLSI breakpoint for *C. albicans*. In addition, clinical PK/PD studies have shown that voriconazole *f*AUC/MIC of <25 was associated with clinical success rates of 52 to 60%, while for patients with an estimated free-drug AUC/MIC of ≥ 32, the success rate has reached 80% (16, 17). Based on the 72-h PK/PD target of 133/94 $f\text{AUC}_{24}$/MIC found in the *in vitro* model for EUCAST/CLSI, a breakpoint of 0.03 mg/liter was determined for both reference methods. The breakpoint of 0.03 mg/liter is exactly at the corresponding ECOFF/ECV (0.03 mg/liter). Isolates with EUCAST/CLSI MICs of 0.06 to 0.125 mg/liter could be classified as I (susceptible, increased exposure) (EUCAST terminology)/SDD (susceptible-dose dependent) (CLSI terminology), whereas isolates with MICs of ≥ 0.25 mg/liter could be classified as resistant. Moreover, associating trough levels with MICs for attaining the PK/PD targets, we found that *C. albicans* isolates with EUCAST/CLSI MICs of 0.06 to 0.125 mg/liter would require trough levels of 1 to 4 mg/liter. Of note, however, 26% of samples in a real-world clinical setting were below 1 mg/liter in a recent study (18), suggesting that such an approach is only feasible if rapid therapeutic drug monitoring service is available and that a significant proportion of the patients will depend on dose escalation before voriconazole monotherapy is appropriate. In contrast, isolates with higher MICs would require trough levels of ≥ 4 mg/liter, which are not feasible to attain and at the same time associated with increased risk of toxicity. In 12 patients with fluconazole refractory candidiasis treated with voriconazole, isolates with CLSI MICs up to 0.39 mg/liter were treated successfully with voriconazole serum trough levels of 2.12 to 4.8 mg/liter (19, 20). This further supports the proposed susceptibility breakpoints and target values from therapeutic drug monitoring.

The $\sim$100 *f*AUC/MIC is similar to the PK/PD target associated with treatment success in invasive candidiasis for fluconazole (21, 22) and provides PTAs that corroborate current clinical susceptibility breakpoints. In addition, the clinical response of *C. albicans* infections to voriconazole therapy has been reported to be 73% for isolates in which the MIC is ≤ 0.008 mg/liter in a large data set (79 patients) (23), whereas the number of patients infected with isolates with higher MICs were too low (<3 patients per MIC) for drawing any conclusions on voriconazole efficacy against those isolates. A recent multicenter study showed that Etest and EUCAST generate similar MICs for voriconazole (mode/ECOFF, 0.008/0.03 mg/liter) although with wide distributions (6, 24). Moreover, in a clinical study with 44 patients with invasive fungal diseases, most with candidiasis ($n = 31$) by *C. albicans* ($n = 17$) where MICs were determined with Etest, the mean trough/MIC ratio associated with clinical efficacy was 11.33, which is close to the trough/MIC ratio of 8 found in the present study (25). These findings suggest that the suggested breakpoints are applicable for Etest endpoint interpretation provided the performance in the laboratory is confirmed by modal MICs for *C. albicans* of 0.008 mg/liter. Mutations in ERG11 and overexpression of multidrug resistance (MDR)/*Candida* drug resistance (CDR) pumps were found in *Candida glabrata* isolates with MICs of ≥ 0.06 mg/liter (23, 26). Introduction of ERG11 alleles in azole-susceptible *C. albicans* isolates indicated a correlation between fluconazole MICs of 0.25, 2, and 4 mg/liter with voriconazole MICs of 0.008, 0.06, and 0.125 mg/liter, further supporting the susceptibility breakpoint of 0.03 for voriconazole considering the fluconazole susceptibility breakpoint of 2 mg/liter and the higher pharmacokinetic variability of voriconazole (27). Epidemiological studies have shown that 44/44 *C. albicans* isolates with MICs $>$ ECVs (0.03 mg/liter) have mutations associated with acquired resistance (28).

The clinical significance of the chosen EI_{50} endpoint, which corresponds to an ~2-log_{10} CFU/ml decrease from drug-free control at 72 h (and also increase from initial inoculum) for azoles and *Candida* species, is unknown. Usually, stasis or 1 log kill is used, although again with no solid support for the clinical significance of those effects. The 2 log_{10} CFU/ml lower growth compared to that of the drug-free control is further supported by the clinical AUC/MIC of 100 for fluconazole (21, 22), which corresponds to a 2 log_{10} CFU/kidney reduction compared to untreated neutropenic animals (~1 log_{10} increase from initial inoculum) (29). One explanation that this may be sufficient and a relevant target might be the absence of neutrophils both in *in vitro* and in neutropenic animal studies that usually contribute to a favorable outcome in patients with invasive candidiasis, particularly in ICU patients. Indeed, *in vivo* studies of experimental invasive candidiasis in neutropenic and nonneutropenic mice showed that median survival was prolonged and fungal load in kidney decreased by 1 log_{10} CFU/kidney, whereas fluconazole reduced further 1 log_{10} CFU/kidney in nonneutropenic mice compared to that in neutropenic mice (30). In addition, voriconazole increased phagocytosis of *Candida* conidia by monocytes/polymorphonuclear leukocytes (31). Thus, the 2-log_{10} CFU reduction in preclinical neutropenic models for azoles may be sufficient for clinical efficacy.

In conclusion, the PK/PD target determined in the present study using a model that was validated based on *in vivo* data from a neutropenic animal model indicated that a CLSI and EUCAST susceptibility breakpoint for *C. albicans* and voriconazole exactly at the respective epidemiological cutoff values of ≤0.03 mg/liter would be appropriate in order to avoid using voriconazole for non-wild-type isolates, which harbor mutations associated with acquired resistance and to further promote the harmonization of the two methodologies.

MATERIALS AND METHODS

***Candida* isolates.** Four *C. albicans* strains previously used in an animal model of disseminated candidiasis were tested (11). The median MICs were determined with the EUCAST methodology using RPMI 1640 with 2% dextrose medium (32) and the CLSI M27-A3 using standard RPMI 1640 medium (0.2% dextrose) (33) in at least triplicate experiments in three different centers. The isolates were stored in normal sterile saline with 10% glycerol at −70°C and revived by subculturing on Sabouraud dextrose agar (SDA) plates supplemented with gentamicin and chloramphenicol (SGC2; bioMérieux) to ensure purity and viability. Inoculum suspensions were prepared in normal sterile saline from 24-h cultures and adjusted to a final inoculum of 10^4 CFU/ml using a counting chamber. The CFU number was confirmed by quantitative cultures on SDA plates.

Antifungal drugs and medium. Pure powder of voriconazole (Pfizer, Inc., Athens, Greece) was dissolved in sterile dimethyl sulfoxide (DMSO) (Carlo Erba Reactifs SDS, Val de Reuil, France), and stock solutions of 10 mg/ml were stored at −70°C until use. The medium used throughout was RPMI 1640 medium (with L-glutamine, without bicarbonate) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (AppliChem GmbH, Darmstadt, Germany) and supplemented with 100 mg/liter chloramphenicol (AppliChem GmbH, Darmstadt, Germany).

***In vitro* PK/PD model.** A previously described two-compartment PK/PD diffusion/dialysis model simulating *in vivo* pharmacokinetics (10) was used. The model consists of an external compartment (EC) comprised of a conical flask connected to a peristaltic pump (Minipuls Evolution; Gilson, Inc.) and an internal compartment (IC) comprised of a 10-ml-volume semipermeable cellulose dialysis tube (molecular weight < 20 kDa, Spectra/Por Float-A-Lyzer G2; Spectrum Laboratories, Inc., Breda, The Netherlands), inoculated with 10^4 CFU/ml yeast suspension. Repeated sampling of 100 μl was made from the IC in order to assure that drug concentrations in the IC indeed mimic voriconazole drug concentration profiles in human or animal plasma. Samples were stored at −70°C until tested. Replicate experiments were conducted in order to assess the reproducibility.

***In vitro*/*in vivo* correlation.** The *in vitro* PK/PD model was validated using the 4 *C. albicans* isolates previously used in a neutropenic murine candidiasis model (11). The voriconazole mouse plasma concentration-time profiles of 10, 20, and 40 mg/kg once daily in mice were simulated in the *in vitro* PK/PD model targeting maximum mouse plasma concentrations (C_{max}) of mean ± SD of 0.47 ± 0.10, 1.67 ± 0.69, and 6.9 ± 2.40 mg/liter and area under the 24-h time-total drug concentration curves ($tAUC_{0-24}$) of mean ± SD of 0.72 ± 0.12, 3.84 ± 1.70, and 27.2 ± 12.2 mg · h/liter, respectively, with an average half-life of 0.9 ± 2.9 h. Drug concentrations were added at the corresponding C_{max} values in the *in vitro* model once daily for 2 days. The log_{10} CFU per milliliter (log_{10}CFU/ml) and voriconazole levels were determined at regular time intervals as described below. The 24-h change in log_{10}CFU/ml compared to initial inoculum at $t = 0$ h versus $fAUC_{0-24}$/MIC relationship was analyzed with the Emax model, and the $fAUC_{0-24}$/MIC associated with 50% of maximal activity was estimated and compared with the *in vivo* $fAUC_{0-24}$/MIC associated with 50% of maximal reduction of fungal load in mouse kidneys after 1 day of treatment (11). Furthermore, the pharmacodynamic effects after 48 h were also studied and the 48-h change in log_{10} CFU per milliliter versus $fAUC_{0-24}$/MIC relationship was analyzed with the Emax

model. For comparison with the *in vivo* PK/PD data, the 22% unbound fraction of voriconazole in mouse serum was taken into account (11).

***In vitro* pharmacokinetics.** Different voriconazole drug concentration-time profiles were simulated in the *in vitro* PK/PD model, with fC_{max} values of 28, 7, 1.75, 0.8, and 0.008 mg/liter and a half-life of 6 h. Voriconazole levels were measured using a microbiological agar diffusion assay as previously described using a voriconazole-susceptible *Candida parapsilosis* isolate (34). The lowest limit of detection was 0.25 mg/liter and intra/interday variation of <15%. The data obtained were subjected to nonlinear regression analysis based on the one-compartment model described by the equation $C_t = C_o e^{-k/t}$ where C_t (dependent variable) is the drug concentration at a given time t (independent variable), C_o is the initial drug concentration at 0 h, e is the physical constant 2.18, and k is the rate of drug removal. The half-life was calculated using the equation $t_{1/2} = 0.693/k$ and compared with the respective values observed in humans. Finally, the area under the dosing interval time-free drug concentration curves (fAUC$_{0-24}$) was calculated for each simulated dosage by applying the trapezoidal rule (the fAUC for fC_{max} 0.008 mg/liter was extrapolated).

***In vitro* pharmacodynamics.** To estimate the fungal load inside the dialysis tubes (internal compartment) of each voriconazole dosing regimen, 100-μl samples were collected at regular intervals up to 72 h, 10-fold serially diluted in normal saline, and subcultured on SAB plates. Plates were incubated at 30°C for 24 h, and colonies were counted at each dilution. Dilutions that yielded 10 to 50 colonies were used in order to determine the $\log_{10}$ CFU per milliliter at each time point and construct the time-kill curves. The lowest limit of detection was 1 $\log_{10}$ CFU/ml.

PK/PD modeling. To determine the *in vitro* exposure-response relationship, the $\log_{10}$CFU/ml at 72 h was subtracted by $\log_{10}$CFU/ml at a t of 0 h and plotted over fAUC$_{0-24}$/MIC ratio for each simulated dose and isolate. The data were then analyzed with nonlinear regression analysis using the sigmoidal model with variable slope (E_{max} model) described by the equation $E = (E_{max} - E_{min}) \times EI^n/(EI^n + EI_{50}^n) + E_{min}$, where E_{max} is the maximum increase $\log_{10}$ CFU per milliliter in the drug-free control (kept contact to $\log_{10}$ CFU per milliliter in drug-free control), E_{min} is the minimum $\log_{10}$ CFU per milliliter found at high drug exposures (kept constant to -1 $\log_{10}$ CFU/ml), the EI is the exposure index fAUC$_{0-24}$/MIC, EI_{50} is the exposure index required to achieve 50% of E_{max}, E_{min}, and n is the slope of the dose-effect relationship (Hill coefficient). The goodness of fit of the E_{max} model was assessed by visual inspection of graphs, residuals analysis, post run's test, and R^2. All data were analyzed using the statistics software package GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA).

Monte Carlo simulation. Monte Carlo simulation analysis was performed using the normal random number generator function of an Excel spreadsheet (MS Office 2007) for 1,000 patients receiving the standard intravenous voriconazole dosage of 4 mg/kg intravenously (i.v.) or 300 mg orally twice daily, which corresponds to a total mean ± SD tAUC$_{0-12}$ of 50.4 ± 41.83 mg · h/liter (12). For the simulation analysis, the fAUC$_{0-24}$ was calculated as 2× fAUC$_{0-12}$, where the fAUC$_{0-12}$ was 21.4 ± 17.57 mg · h/liter based on the 42% unbound fraction of voriconazole in human serum (35). The probability of target attainment (PTA) for EI_{50} was estimated for isolates with MICs ranging from 0.008 to 4 mg/liter, and PK/PD susceptibility breakpoints were determined. The 95% CI of each PTA was calculated based on the upper and lower 95% CI limit of EI_{50}. Previously published MIC distribution data for *C. albicans* with CLSI (28) and EUCAST (6) were used. The MICs for which the lower 95% CI limit was higher than 95% and 50% PTA were determined.

Trough levels and MICs. The required trough levels in human serum necessary to attain the mean and 95% CI limits of EI_{50}s were calculated for different MICs. For that reason, the previously described relationship between serum tAUC and trough concentrations (tC_{min}), namely, tAUC$_{0-12} = 7.011 + 12.687 \cdot tC_{min}$ (36) was used, taking into account the 42% unbound fraction of voriconazole in human serum (13). The EUCAST and CLSI24 MICs for *C. albicans* at which the corresponding PK/PD targets were attained were plotted against the tAUC$_{0-12}$ and tC_{min}.

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